การใช้กาวไฟบรินจากเลือดกระบือเพื่อตรึงเยื่อหุ้มรกมนุษย์ในการรักษาแผลหลุมบนกระจกตาในกระต่ายทดลอง

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SUTURELESS HUMAN AMNIOTIC MEMBRANE FIXATION USING BUBALINE FIBRIN GLUE FOR CORNEAL SURFACE RECONSTRUCTION IN EXPERIMENTAL RABBITS.

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ภู่ภัทร แก้วยอดจันทร์ : การใช้กาวไฟบรินจากเลือดกระบือเพื่อตรึงเยื่อหุ้มรกมนุษย์ในการรักษาแผลหลุมบนกระจกตาในกระต่ายทดลอง (SUTURELESS HUMAN AMNIOTIC MEMBRANE FIXATION USING BUBALINE FIBRIN GLUE FOR CORNEAL SURFACE RECONSTRUCTION IN EXPERIMENTAL RABBITS.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. สุมิตร ดุรงค์พงษ์ธรสพ.บ., Ph.D., อว.สพ.สาขาศัลยศาสตร์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. วิจิตร บรรลุนาราสพ.บ., Ph.D., อว.สพ.สาขาพยาธิวิทยา {, 47 หน้า.

การศึกษานี้เป็นการศึกษาประสิทธิภาพของกาวไฟบรินที่ผลิตจากเลือดกระบือเปรียบเทียบกับการใช้วัสดุผูกเย็บในการทำศัลยกรรมตรึงยึดเยื่อหุ้มรกจากมนุษย์บนกระจกตาของกระต่ายทดลอง เลือดกระบือเก็บจากกระบือสุขภาพดีด้วยวิธีปลอดเชื้อ ผ่านกระบวนการตกตะกอนด้วยความเย็นเพื่อแยกให้ได้สารสกัดไฟบริโนเจนและสารสกัดทรอมบิน การทดลองทำในกระต่าย 10 ตัว แบ่งเป็น 2 กลุ่ม กลุ่มควบคุมใช้การตรึงยึดด้วยวัสดุผูกเย็บชนิดไนลอนจำนวน 3 ตัว กลุ่มทดลองใช้การตรึงยึดด้วยกาวไฟบรินจากเลือดกระบือจำนวน 7 ตัว เก็บข้อมูลเวลาที่ใช้ในการผ่าตัด กระต่ายถูกเลี้ยงเพื่อดูอาการ 14 วัน และบันทึกข้อมูลการย้อมติดสีฟลูออเรสเซนส์ ระดับน้ำตา ความใสของกระจกตา ความเจ็บปวด และ อาการทางคลินิก หลังจากทำการุณยฆาต ลูกตาถูกผ่าตัดเพื่อนำไปผ่านกระบวนการทางจุลพยาธิวิทยาเพื่อศึกษารูปแบบของการหายของบาดแผลในแต่ละกลุ่มการทดลอง ผลการทดลองพบว่า ระยะเวลาที่ใช้ในการผ่าตัดของกลุ่มทดลองน้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ กระจกตาในกลุ่มทดลองมีคะแนนความใสดีกว่าในกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ และรูปแบบการเข้ามาปกคลุมบาดแผลของเซลล์เยื่อบุผิวในกลุ่มทดลองมีรูปแบบที่เป็นระเบียบมากกว่า เยื่อหุ้มรกในกลุ่มทดลองแนบชิดไปกับผิวกระจกตาดีกว่าในกลุ่มควบคุม ในขณะที่เยื่อหุ้มรกในกลุ่มควบคุมมีการบวมน้ำและขอบม้วน ทำให้การเข้ามาของเซลล์เยื่อบุผิวไม่เป็นระเบียบ การใช้กาวไฟบรินจากเลือดกระบือ ให้ผลการรักษาทางคลินิกที่ดีกว่าและใช้เวลาในการผ่าตัดน้อยกว่าการใช้วัสดุผูกเย็บ

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POUPAT KEAWYODJAN: SUTURELESS HUMAN AMNIOTIC MEMBRANE FIXATION USING BUBALINE FIBRIN GLUE FOR CORNEAL SURFACE RECONSTRUCTION IN EXPERIMENTAL RABBITS.. ADVISOR: ASST. PROF. SUMIT DURONGPHONGTORN, D.V.M., D.V.Sc., D.T.B.V.S., CO-ADVISOR: ASSOC. PROF. WIJIT BANLUNARA, D.V.M., Ph.D., D.T.B.V.P.{, pp.

This study was designed to compare the efficacy of the buffalo-blood fibrin glue or bubaline fibrin glue with the suture material for the fixation of the human amniotic membrane on the corneal surface in experimental rabbits. Bubaline blood was drawn aseptically and processed by cryoprecipitation to extract fibrinogen and thrombin. Ten of rabbits were divided into 2 groups, the control (n=3) and the treatment (n=7). The membrane was fixed with Nylon and the bubaline fibrin glue in the control and the treatment groups respectively. The surgical time was recorded. Animals were reared for 14 days and collected the duration of the presence of fluorescein staining, tear production, corneal clarity score, pain score and clinical outcomes. The animals were euthanized and the eyes were enucleated. The eyes were histopathologically processed for wounding study. The surgical time and corneal clarity score in the treatment group was shorter than the control group significantly *(P<0.05).* Epithelialization pattern in the treatment group was more organized than that in the control group, and the membrane was attached more closely than the control group. Use of the bubaline fibrin glue could provide better clinical outcomes and less surgical time than the suture materials.

Department:	Veterinary Surgery	Student's Signature	.....
Field of Study:	Veterinary Surgery	Advisor's Signature	.....
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CHAPTER I

INTRODUCTION

Importance and Rationale

Corneal ulceration, loss of corneal epithelium and corneal stromal layer, is one of the major problems of the cornea that affects the vision and causes several secondary problems including anterior uveitis, corneal perforation, secondary glaucoma and blindness. Medical management including topical eye drops and oral medications should be given as soon as possible to limit the progression of the disease. If the defect is complicated or too large to be managed with the medications, surgery must be initiated promptly.

Human amniotic membrane transplantation (HAMT) is one of the most effective surgical procedures to correct large and complicated cases of corneal ulceration. Many reports showed that human amniotic membrane (HAM) could promote corneal wound healing. Suture materials have been used to secure the membrane tightly on the defected cornea for decades. However, it is a time-consuming procedure and induces an inflammation on the cornea due to suture material – patient tissue reaction (Szurman et al., 2006). Panda et al. (2009) supported that suture materials induced corneal irritation and inflammation; moreover, it could cause postoperative wound infection and graft rejection. Fibrin glue has been introduced to the field of ophthalmic surgery to solve the limitations.

Fibrin glue is a biological degradable adhesive consisting of 2 major components, fibrinogen and thrombin. Once the components are mixed, a final step of coagulation cascade occurs and forms a clot. Szurman et al. (2006) reported that using fibrin glue instead of the suture materials decreased the suture material – patient tissue reaction and overall surgical time. The amniotic membrane fixed with the fibrin glue remained attached on the cornea, while the suture-fixated membrane lost some of the membrane and had a prominent edge with epithelial cells growing underneath.

Fibrin glue has been launched commercially in several different trade names. All are derived from the same sources of blood; fibrinogen from bovine blood and thrombin from human pooled blood. According to cryoprecipitation technique, Thomazini-Santos et al. (1998) showed that buffalo's blood had the highest level of fibrinogen at 664 mg/dl when they had 375.5 mg/dl in human, 267 mg/dl in ovine, 240 mg/dl in equine and the lowest in bovine at 218.33 mg/dl. Although buffalo's blood provides the highest level of fibrinogen, knowledge of buffalo's blood derived fibrin glue is limited and use of the commercial fibrin glue is costly.

Objective of the Study

The study is designed to compare the efficacy of the bubaline fibrin glue with suture material on fixing the HAM in treating the artificial corneal ulcer in experimental rabbits.

Research questions

1) Could the bubaline fibrin glue fix HAM as securely as or better than the suture material on the experimental rabbit wounded cornea?
2) Could the bubaline fibrin glue minimize complications and provide better outcomes than the suture material?

Advantages of the study

Fibrin glue has been widely used in medical field but not in the veterinary field. This study will encourage the use of bubaline fibrin glue in the veterinary field, especially, veterinary ophthalmology. Furthermore, this study would raise buffalo farming in Thailand for producing the biomaterials in the medical and veterinary industries.

CHAPTER II

REVIEW OF LITERATURE

Anatomy and physiology of the cornea

Cornea is an outermost transparent structure of the eye. It is a load-bearing tissue whose primary function is to allow light to refract and pass through the eyeball forming an image on a retina and also protect an intraocular content. The cornea is normally clear, avascular and non-pigmented. In domestic animals, the horizontal diameter of the cornea generally greater than that in the vertical diameter, but this characteristic will be notable obviously in herbivorous species. The cornea is thickest at the periphery and gradually thinner to the center. The corneal thickness is changeable during a day and varies among species but it is usually not greater than 1.00 mm. In rabbits, it is 355±6 microns thickness at the central cornea (Chen et al., 2011).

Four important layers of the cornea running outward to inward consist of stratified epithelium, collagenous stromal layers, descemet's membrane and endothelium (Figure 1).

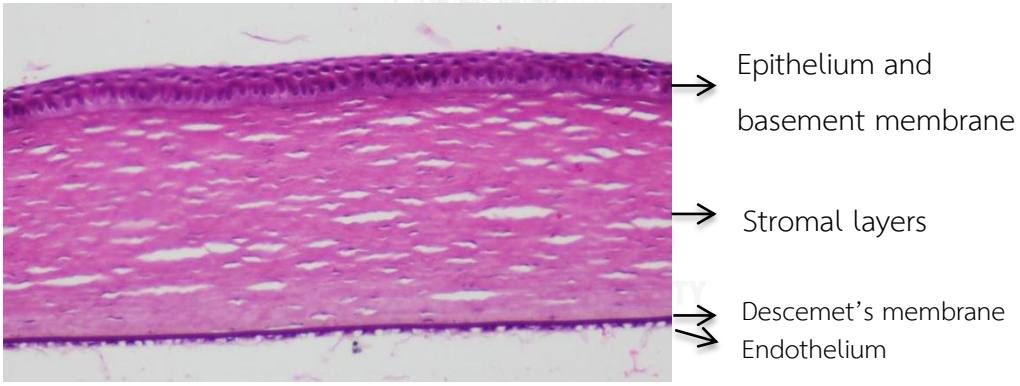

Figure 1 Normal histological structure of rabbit's cornea; epithelial layers with basement membrane, collagenous stromal layers, descemet's membrane and endothelium

The corneal epithelium, from deep to superficial, comprises the basal cells, wing cells and squamous surface cells. Basal cells are columnar attaching on thin basement membrane. Its turn over time is approximately 7 days and this characteristic provides the epithelium being a well regenerative tissue. The wing cells are polygonal divided from basal cell and forced toward the surface. Basal cells are replaced by the limbal stem cells.

The corneal stroma is the thickest layer of the cornea, 90%, which consist of keratocyte, collagen fibrils and ground substance. Keratocyte produces and maintains the stromal lamellae. The formation of scar tissue on the cornea may be related with the keratocytes since they can transform themself into myofibroblasts in case of deep corneal injuries (Samuelson, 2013). The lamellae bundles are parallel to each other with each lamella running the entire diameter of the cornea. The specific arrangement of the collagen fibril bundles is the importance in maintaining corneal clarity. Deturgescence is a term to describe normal hydration status of the cornea that is relatively dehydrated compared with other tissue from 75% - 85% water in its tissue. Epithelium and endothelium work on moving sodium and calcium ions out of the corneal stroma into tear and aqueous humor by energy-dependent sodium-potassium ATPase pumps. Removal of the epithelium results in corneal edema and produces corneal thickness up to 200% after 24 hours and. Removal of endothelium produces an increase of 500% or more in corneal thickness (Samuelson, 2013).

Descemet's membrane is a basement membrane of the endothelium. It is homogenous, acelluar, thin membrane forming an inner protective boundary between the stroma and the endothelium. The descemet's membrane is continuously secreted by the endothelium throughout life, this membrane thickens with age.

Corneal endothelium is a single cell layer lining the innermost of cornea and exposes aqueous humor. Its role is to pump ions from the stroma into the aqueous and the movement of water follows these ions ensuring that the cornea remains dehydrated. Endothelial cells' capacity is limited to replicate in adult animals. In senile age, endothelial cells are lost and the corneal stroma becomes thicker owing to edema.

Because the cornea is avascular, oxygen, nutrients and metabolites must be obtained and disposed through alternate route; precorneal tear film, aqueous humor, atmosphere and adjacent capillaries. Anterior cornea takes most of nutrients and oxygen from the precorneal tear film and atmosphere, and the aqueous humor is major source of posterior cornea.

Corneal wounding

Studies of corneal wound healing have been examined for a long history; thus, methods of wounding cornea have been developed along. The review of wounding cornea to learn how it heals by Stepp et al. (2014) informed that there are several methods to produce a corneal wound in different types of wound; circular

debridement wounds, rotating burr circular wounds, manual superficial keratectomy (MSK) and photorefractive keratectomy (PRK) wounds, incisional wounds, pocket assay and suture wounds, and filter paper or impression injuries. Either method has its own properties to use for study how cornea heals itself. MSK is a wound of choice for studying corneal scarring. MSK is created by trephination, apply on the cornea and rotate with slightly pressure, and remove a corneal button. The wound healing occurring after this procedure induces re-epithelialization, basement membrane reassembly, re-innervation, and corneal scarring.

Healing process of the cornea

Healing process of the cornea is a complex dynamic cascade to bring a normal function of the tissue and maintain the clarity of the cornea. Because there are 3 different types of cell layers, each layer has its own way to reconstruct.

1) Keratocyte apoptosis

Keratocyte apoptosis is the earliest event occurring immediately underneath the defect after the epithelium injury. This process plays the important role of corneal wound healing by triggering subsequent cellular process(Wilson et al., 2003; Wilson et al., 2007). IL-1 and TNF-α released from the damaged epithelial cells modulate apoptosis of keratocytes (Wilson et al., 2001; Wilson et al., 2003). Wilson and colleagues proposed that the process is developed to limit spreading of the pathogen; especially viral pathogen, not to penetrate to the posterior cornea and protect the cornea from excessive releasing of the cytokines and others protein that would destroy the normal structure. Simultaneously, the apoptosis cells produce cytokines moving upwards to stimulate function of the surface epithelium.

2) Epithelial healing process

In the normal cornea, the basal cells of the epithelium are well-mitotic cells. The daughter cells divided from the basal cells move anteriorly and gradually differentiate into the superficial epithelial cells and replace outermost layer of epithelium. The cycle takes approximately 1-2 weeks (Suzuki et al., 2003). The supply of corneal epithelial cells depends on limbal stem cells, but if the limbal stem cells intact; the closure of epithelial defect depends on only basal cells. Presuming the limbal epithelial cells is intact, once the epithelium is insulted, the neighboring epithelial cells immediately migrate to cover the defects with a monolayer and epithelium locating far from denuded area begins mitosis. After complete epithelial covering, the

cells begin proliferation to restore the normal thickness of the cornea; however, it is not appropriately. A few weeks later, the cells begin to differentiate and the surface become smooth and restores the normal anatomy of the surface of cornea. Thus, three phases of corneal epithelial wound healing are composed of cell migration, cell proliferation and cell differentiation (Suzuki et al., 2003).

3) Corneal stromal healing process

The healing of stroma is quite different from the epithelium. Keratocytes lying in the stroma are separated from another by extracellular matrix (ECM); collagen fibrils and glucosaminoglycans (GAGs), is well-known as the key of corneal transparency (Dayhaw-Barker, 1995b). As previous document, keratocyte apoptosis is the first event following the disruption of the cornel epithelium, it appears to continue extending for at least 1 week (Wilson et al., 2001), and the histological changes can be observed within 30 minutes. For those at the margin of the wound, the cells dedifferentiate back to fibroblast, proliferate and begin to migrate into the wounded area within 2 days and peaks between 3-6 days (Dayhaw-Barker, 1995a). This process is followed by the reestablishment of collagen, proteoglycans and other substances in the wounded area. Under normal conditions, the stromal collagens are mainly Types I, III, V, and VI. Several studies have examined which types of collagen is mainly synthesized after keratectomy, Dayhaw-Barker (1995) had gathered the reports and stated that Type III collagen is synthesized within days after injury. The process of stromal remodeling and repair is long lasting for 3 months or above.

4) Endothelial healing process

For endothelium, the studies indicated that the normal endothelial cell begin to extend its cell membrane to be a pseudopodia-like extensions and migrate to wounded area. After recovering the defect, the cells start proliferating themselves to fulfill the denuded area.

Human amniotic membrane transplantation (HAMT)

Human amniotic membrane (HAM) is composed of 3 layers; epithelial cell layer, basement membrane layer, and connective tissue membrane (Sippel et al., 2001; Dua et al., 2004). In 1940, Davis had been reported the first use of HAM for surgical skin transplantation (Dua et al., 2004; Baradaran-Rafii et al., 2007). The earliest use of HAM in ophthalmic surgery dates back to 1940, Rotth and Sorsby used fetal

membrane (both chorion and amnion) to treat conjunctival defect (Fernandes et al., 2005). After that, the use of HAM in ophthalmology had been paused until Kim and Tseng in 1995 reintroduced it back to ophthalmology by applying non-cryopreserved HAM on the diseased eye of the experimental rabbits (Baradaran-Rafii et al., 2007).

Applying HAM on the ocular surface, HAMT, is beneficial. Several studies showed the strength of HAM. Baradaran-Rafii et al. (2007) indicated that it could reduce inflammation and scarring, enhanced wound healing and epithelialization and it had antimicrobial properties. Gomes et al. (2005) informed three basic properties of HAM; there were anti-inflamatory effect, antiscarring effect and neurotrophic factors, and antiangiogenesis. Recent study, Lockington et al. (2014) performed a pilot study to answer whether HAM could remove reactive oxygen species (ROS) from the environment and the result was desirable, HAM could prevent the destruction of corneal cells from the ROS.

Surgical techniques of HAMT

The HAM is fixed on the ocular surface with its epithelium sides up. Cryopreserved HAM is prepared by mounting on the nitrocellulose paper with basement membrane face up. Its preparation helps surgeons are easy to recognize which side it is. After removing of necrotic tissue and loose epithelium from the defect, the HAM is spread onto the surface avoiding air, blood or fluid entrapped underneath and sutured the membrane with polyamide or polyglactin-910 suture (Dua, 1999; Sippel et al., 2001; Fernandes et al., 2005; Baradaran-Rafii et al., 2007).

1) Inlay technique

The membrane acts as the scaffold allowing migration and growth of epithelial cells on it. The membrane is spread on the defect and cut to appropriate size, slightly larger than the defect and sutures with the rim of the corneal wound.

2) Overlay technique

The membrane is spread over the entire cornea including limbus and paralimbal area (Sippel et al., 2001). The membrane is anchored with the limbus and midperiphery of the cornea (Baradaran-Rafii et al., 2007). The membrane acts as the bandage contact lens (BCL) and provides a barrier to protect the defect from the environment, inflammatory cells and tear proteins (Fernandes et al., 2005).

3) Multilayered technique

This technique is suitable for deep ulceration. Multiple pieces of amniotic membrane are used to fill up the defect. The most upper sheet should be placed with the basement membrane side up.

Although transplantation of HAM on denuded cornea provided many desirable outcomes, use of suture materials to secure the graft gives unpleasant outcomes also. Suture placed on the cornea induced an immune response and led to an induction of blood vessels (Stepp et al., 2014). Panda et al. (2009) stated that suture induced irritation and redness of the cornea. Similarly, Takaoka et al. (2008) informed that suture caused post-operative pain and discomfort in human patients, and also was associated with suture-related complications such as, suture abscesses, granuloma formation, and tissue necrosis.

Fibrin glue (FG)

Fibrin glue is a biological degradable adhesive consisting of 2 major components; fibrinogen and thrombin. Once the components is mixed up, a final stage of coagulation cascade occurs and forms a clot. Since the first use of plasma powder as a source of fibrinogen to establish hemostasis in 1909 by Bergel (Radosevich et al., 1997), the fibrin glue has been extensively developed until now and widely used in many medical purposes (Figure 2).

Fibrin glue has been used in several fields of surgery including skin and reconstructive surgery, cosmetic surgery, head and neck surgery, cardiovascular surgery, gastrointestinal surgery, dental surgery and also ophthalmic surgery (Brennan, 1991; Mintz et al., 2001). Sealing corneal perforation, eye lid surgery, conjunctival repairing, pterygium surgery, lens surgery, refractive surgery and corneal reconstruction are the surgeries of the eye that has a report of fibrin glue using (Radosevich et al., 1997; Chan and Boisjoly, 2004; Panda et al., 2009).

The action of fibrin glue mimics a common cascade of coagulation, the last step of blood clotting. Once the coagulation cascade is triggered, prothrombin is activated by factor X and converts to thrombin. The presence of thrombin is necessary for converting of fibrinogen to fibrin; simultaneously, thrombin also activates factor XIII which stabilizes the clot by promoting polymerization of fibrin with assistance of Ca^{2+}. Finally, the complete fibrin clot has formed as glue.

The fibrin clot is naturally degraded by proteolytic enzymes over the time. To prolong function of fibrin glue, antiproteolytic agents should be added into the

preparation. Aprotinin is the proteolytic agent added into the mixture in commercial process to ensure function of the clot last long enough. Tranexamic acid, as well as epsilon aminocaproic acid, at a concentration of greater than 10 mg/ml, have also been used mostly in homemade preparation (Radosevich et al., 1997).

The use of fibrin glue in HAMT

According to the disadvantages of the suture materials, fibrin glue had been introduced to solve the problems. There were several studies of comparison between suture and fibrin glue. Szurman et al. (2006) found that all membranes remained attached in fibrin glue-fixated group with no evidences of graft shrinkage and edema and it was still transparent, while suture-fixated membrane had edge bulging and graft shrinkage, local irritation of the cornea had been recognized, and the membrane was slightly opaque. The researchers also revealed the histological features that the membrane adhered closely to the corneal surface in fibrin glue group, no stromal edema or inflammatory cells and keratocytes and fibrin layers was unaltered. In suture group, a granulomatous reaction with surrounding lymphocytes and epitheloid cells had been reported.

Vichare et al. (2013) performed a study of conjuntival autograft after pterygium surgery in human, patients had been divided into suture group and fibrin glue group. The mean surgical time was significantly shorter in fibrin glue group than suture group, and pain was present in all 30 patients in suture group, but only 14/30 patients in fibrin glue group. The result was related to the study of Mahdy and Wagieh (2011) that there was statistically significant decrease operation time in fibrin glue group. Additionally, both ocular pain and discomfort were less in fibrin glue group. Complications had been noted also minimize in the fibrin glue group.

Grafting with suture materials may cause astigmatism. Cho et al. (2013) conducted lamellar keratoplasty on rabbit cornea and placed the amniotic membrane with fibrin glue and suture, the result showed that there was significantly changed in mean keratometry at 4 weeks postoperatively in suture group; however, no significantly changed in fibrin glue group.

CHAPTER III

Materials and Methods

Animals

1) Ten healthy adult New Zealand White rabbits of both sexes were reared at Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The animals were given food and water *ad libitum* in barrier system with 12-hour light on and 12-hour light off. The animals were randomly divided into two groups; the control group (n=3) and the treatment group (n=7).
2) One healthy adult buffalo used for blood collection via jugular vein.

Materials

Human amniotic membrane (HAM)

Cryopreserved human amniotic membrane purchased from Thai Red Cross Eye Bank, Pathumwan, Bangkok, Thailand.

Fibrin glue preparation

The procedure was modified from the original method of Thorn et al. (2004). Three hundred ml of buffalo's blood drawn from a jugular vein was kept in 30 ml of citrate phosphate dextrose (CPD) as an anticoagulant and centrifuged in room temperature at 1,500 rpm for 15 minutes to separate platelet rich plasma (PRP) from fresh whole blood (Figure 2).

Figure 2 Separation of the PRP after centrifuge.

1) Preparation of thrombin extract

Thirty ml of PRP was added 270 ml of citric acid, and then the mixture was centrifuged 3,000 g for 5 minutes at 4°C. The mixture was separated into two parts, a supernatant and a precipitate. The supernatant was discarded and the precipitate was added 1.8 ml of calcium chloride ($CaCl_2$) [0.1 mol/L]. The mixture of precipitate was titrated to adjust pH to 7 by pouring 1.2 ml of sodium bicarbonate ($NaHCO_3$) and $CaCl_2$ to convert prothrombin to thrombin. The product was kept at -20°C (Figure 3).

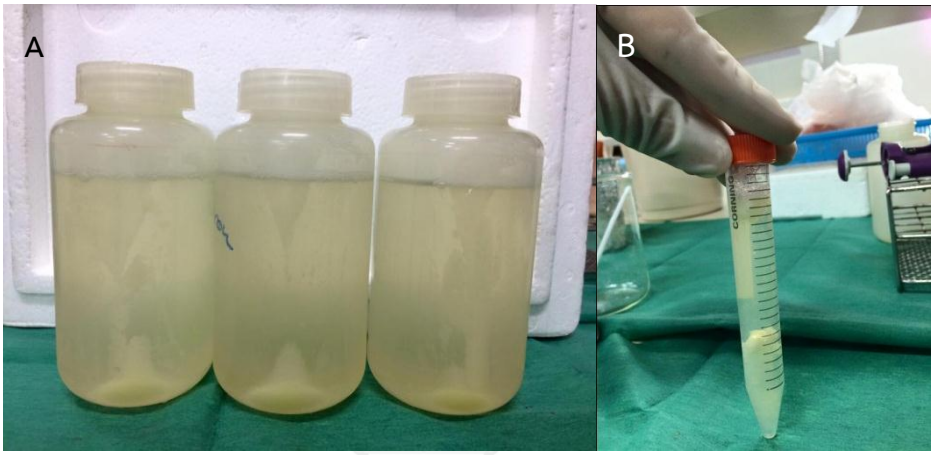

Figure 3 Preparation process of thrombin extract. The precipitate and the supernatant before discarded (A). After pouring of $NaHCO_3$ and left in room temperature for minutes, the solution was kept as thrombin part (B).

2) Preparation of fibrinogen

One hundred ml of PRP was mixed with 3.2 ml of tranexamic acid (10 mg/ml) and 12 ml of chilled absolute ethanol. Then, the mixture was incubated on ice for 20-30 minutes. After the time, the mixture was centrifuged at 3,000 g for 20 minutes at 0-1°C. The supernatant will be discarded, and the precipitate was dissolved in normal saline solution 4-5 ml and kept at -20°C (Figure 4).

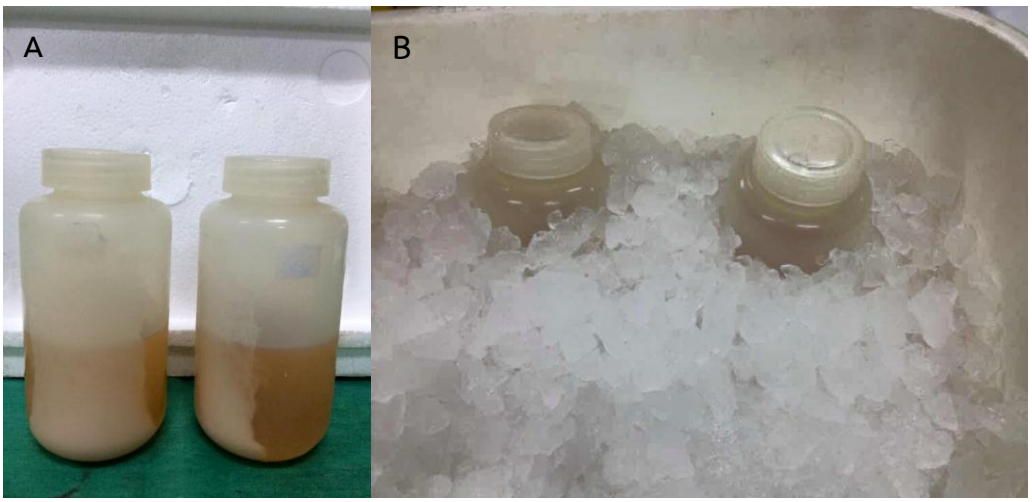

Figure 4 Preparation process of fibrinogen extract. A photograph of the precipitate and the supernatant (A). Incubation on ice of the mixture before the process of cryoprecipitation(B).

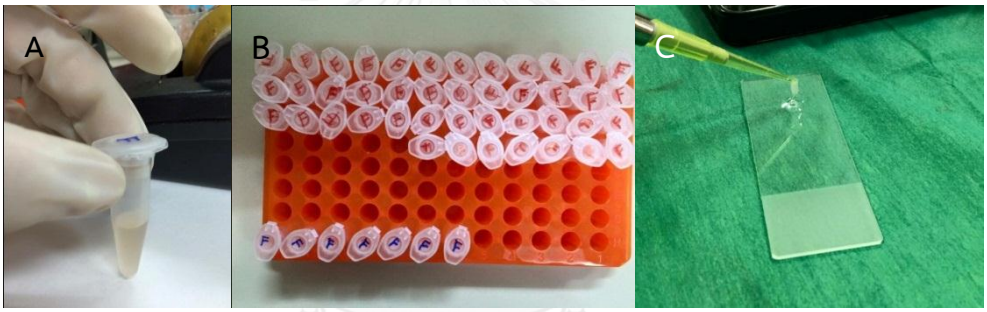

Figure 5 The bubaline fibrin glue. Fibrinogen and thrombin were kept separately in sterile eppendorf tube and freeze at -20°C (A, B). After fibrinogen mixed with thrombin, the glue was formed (C).

Experimental procedures

Pre-operation preparation

All rabbits were performed the complete physical examination and routine ophthalmic examination; ocular response and reflexes tear measurement, fluorescein staining and corneal clarity scoring: prior to the operation.

Operation procedures

1) Anesthesia

The rabbits were premedicated with an intramuscular injection of xylazine HCl (1 mg/kg) and ketamine HCl (25 mg/kg). After endotracheal intubation, general anesthesia was maintained with isoflurane and oxygen supplement. An intravascular catheter was placed in the marginal ear vein and animals

were given lactate ringer solution intravenously throughout the operation. The heart rate, respiratory rate and rectal temperature were monitored until the end of the operation.

2) Corneal wounding

The left eye (OS) of each animal was wounded with 0.4 cm diameter punch biopsy. The punch biopsy was applied on the central cornea and rotated unidirectionally with slightly pressure. A corneal button was removed with an ophthalmic micro-surgical blade (Figure 6).

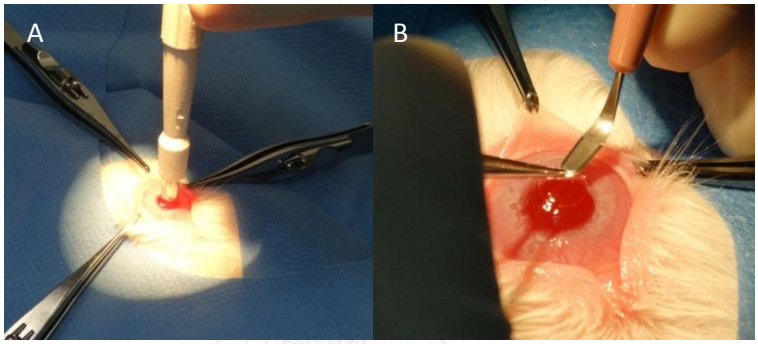

Figure 6 Wounding method; Manual Superficial Keratectomy (MSK). Punch biopsy was put on the central cornea, slightly pressed and rotated unidirectionally (A). A corneal button was removed with an ophthalmic micro-surgical blade (B).

3) Human amniotic membrane transplantation (HAMT)

The animals and surgeons were undergone the aseptic technique before the operation. Inlay technique was used in the study. After insulting the cornea, wound was rinsed with lactate ringer solution and dried. Cryopreserved HAM was aseptically prepared at room temperature, cut the membrane to an appropriate size, slightly larger than the defect, and spread on the cornea with basement side-up (Figure 7). Simultaneously, fibrinogen and thrombin extract were thawed at 37°C. The control group, after applying the membrane properly, 10-0 Nylon was used to anchor the membrane with intracorneal simple interrupted suture pattern for 6 stitches (Figure 8 (A)).

The treatment group; the membrane was soaked with 2-3 drops of fibrinogen. After 3 minutes, the soaked membrane was transferred from the nitrocellular paper to lay onto the recipient eye with basement side-up. After adjusting the membrane, excess fibrinogen was rubbed off and the process was achieved by applying 2-3 drops of thrombin extract and wait until the mixture became whitish (Figure 8 (B)).

The timer started after the membrane adjusted appropriately, and stopped after the last knot tying in the control group and after becoming whitish of the glue in the treatment group (added 3 minutes extra from soaking period). The time was recorded as the surgical time.

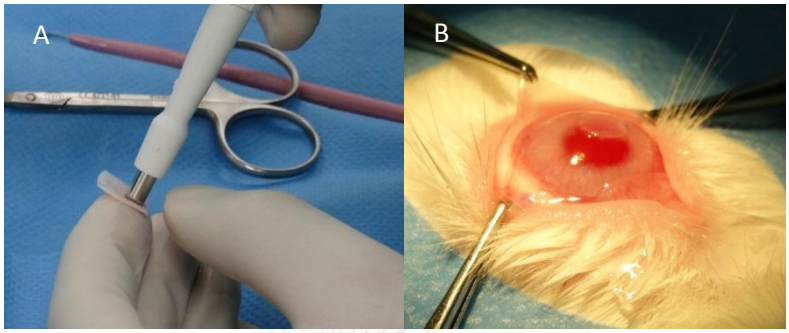

Figure 7 HAM was prepared aseptically before transplantation (A). HAM after transplantation and adjusted appropriately (B).

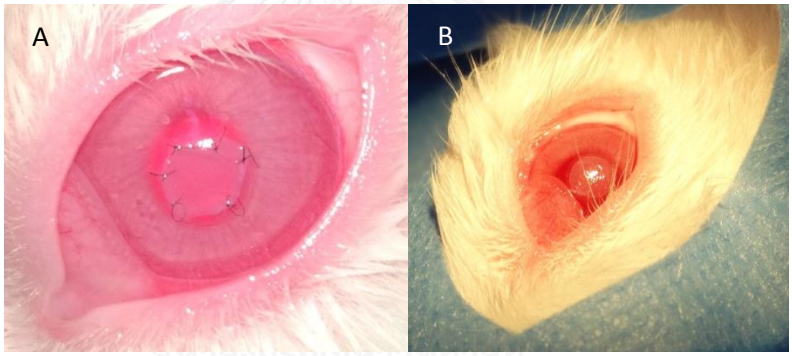

Figure 8 Eyes after the operation. HAM anchored with nylon 10-0 (A). HAM with the bubaline fibrin glue (B).

Post-operative care

The animals were received eye drops including 0.5% atropine, 0.3% tobramycin and artificial tear. Carprofen (4 mg/kg) was subcutaneously injected once a day for the first three days after the operation.

Histopathological and immunohistochemical study

All of animals were euthanized at day 14 after surgery. The eyes were enucleated and immediately fixed in Bouin's solution for 24-48 hours. By the time, the eyes were transfered to be destained in the 10% buffer formalin solution overnight and histologically processed then embedded in the paraffin block. Tissue sections were cut at 4 μm and stained with Hematoxylin and Eosin (H&E) and

Masson's Trichrome to investigate the epithelialization, collagen fibrils, the HAM and inflammatory cells.

Expression of cytokeratin proteins was evaluated by immunohistochemical staining (IHC) with monoclonal antibody against pan-cytokeratin produced in mouse (clone PCK-26) (Sigma-Aldrich, USA). After deparaffinization, tissue was undergone an antigen retrieval method using citrate buffer solution at 60°C. Tissue peroxidase enzyme was blocked with 30% H_2O_2 in methanol and blocked unoccupied antigen site with 8% skim milk at 37°C. After 30 minutes, the tissue was tagged with primary antibody (monoclonal antibody against pan-cytokeratin) and left in a moisture chamber under 4°C overnight. Then, the tissue was tagged with secondary antibody (EnVision™ Polymer DAKO) and developed with DAB chromogen. The last step was counterstained with hematoxylin then dehydrated and mounted with permount media. The positive immunostaining was observed with light microscope.

Data Collection and Evaluation

All eyes were examined with ophthalmic illuminator light for corneal clarity, clinical signs and graft characteristics, fluorescein staining for evaluation of epithelialization and Schirmer tear test (STT) strip for tear production.

1) Graft characteristics: The grafts were observed on day 0, 3th, 6th, 9th and 12th and recorded signs of graft shrinkage, graft folding, graft edema and graft slough. The data was evaluated with descriptive statistics using SPSS program (IBM Corporation, NY, USA).

2) Duration of the epithelialization: The wounds were stained with fluorescein on day 3rd, 6th, 9th and 12th after the operation. The means of the duration were compared by independent student t-test with a significant level at $p<0.05$ using SPSS program (IBM Corporation, NY, USA). Kolmogorov-Smirnov test was used to test the normality.

3) Corneal clarity score

The clarity of corneal was evaluated on day 14th. The corneal clarity scoring system used in the study was modified from the study of Guzey et al. (2001) and Krueger et al. (2008) as shown beneath

Score 1: Clear cornea

Score 2: Mild haze, all of underlying structures are easily seen

Score 3: Moderate haze, some of underlying structures are seen

Score 4: Severe haze, none of underlying structures are seen

The data was compared between the groups by non-parametric; Mann-Whitney U test, with a significant level at $p<0.05$ using SPSS program (IBM Corporation, NY, USA).

4) Pain scoring

The scoring followed the rabbit grimace scale of National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs, London, England)

Score 0: Normal present

Score 1: Narrowing of palpebral fissure

Score 2: Tightly close of the eyelid and wrinkle may be visible

The data was compared between the groups by non-parametric; Mann-Whitney U test, with a significant level at $p<0.05$ using SPSS program (IBM Corporation, NY, USA).

Histopathological evaluation

Histopathology and immunohistochemical studies were described in the term of the epithelialization pattern, the graft characteristic and the presence of the inflammatory cells.

CHAPTER IV
RESULTS

Ten rabbits were randomly divided into two groups. The control group including one female and two male (n=3) was treated with conventional treatment and the treatment group including three female and four male was treated with interventional treatment (Table 1).

Table 1 Number of the animals of the study.

Group of study	Number of animals	Male	Female
Control group	3 (30%)	2 (66.67%)	1 (33.33%)
Treatment group	7 (70%)	4 (57.14%)	3 (42.86%)

Fibrin glue

Bacterial culture

Whole blood, fibrinogen extract and thrombin extract were sent to undertake a bacterial culture and the result was no bacterial growth.

Fibrinogen concentration

The whole blood had 400 mg/dl of fibrinogen level and 2,800 mg/dl in the fibrinogen extract on the first day after producing. After keeping under -20°C, there was 1,400 mg/dl of fibrinogen level on day 30 and 60 post-producing (Table 2).

Table 2 Fibrinogen concentration

	Day 0	Day 30*	Day 60*
Whole blood	400	-	-
Fibrinogen extract	2,800	1,400	1,400

* Keeping under -20 °C

Pre-operation examination results

All rabbits were performed routine examination, normal heart and lung sound with normal rhythm. Gut sound was normal without any signs of gut stasis and bloat. Fecal shape and characteristic were normal indicating the normal function of gastrointestinal system. Teeth were normal aligned without any spurs. Oculo-nasal discharge was not indicated in all animal. Ocular examination showed normal outcomes. Ocular reflexes and responses; blink reflex, PLR and dazzle reflex were

positive in both eyes of all rabbit. Menace response was not indicated in all rabbits. Fluorescein was not stained on every cornea.

Post-operative results and clinical outcomes

Surgical time

Mean surgical time of the control group was 20.14±2.64 minutes which were range from 18.00 – 23.15 minutes and mean surgical time of the treatment group was 3.16±1.46 minutes which was ranged from 2.20-6.45 minutes. The data was normally distributed and there was a statistically significant difference between the groups (*p<0.05*) (Figure 9).

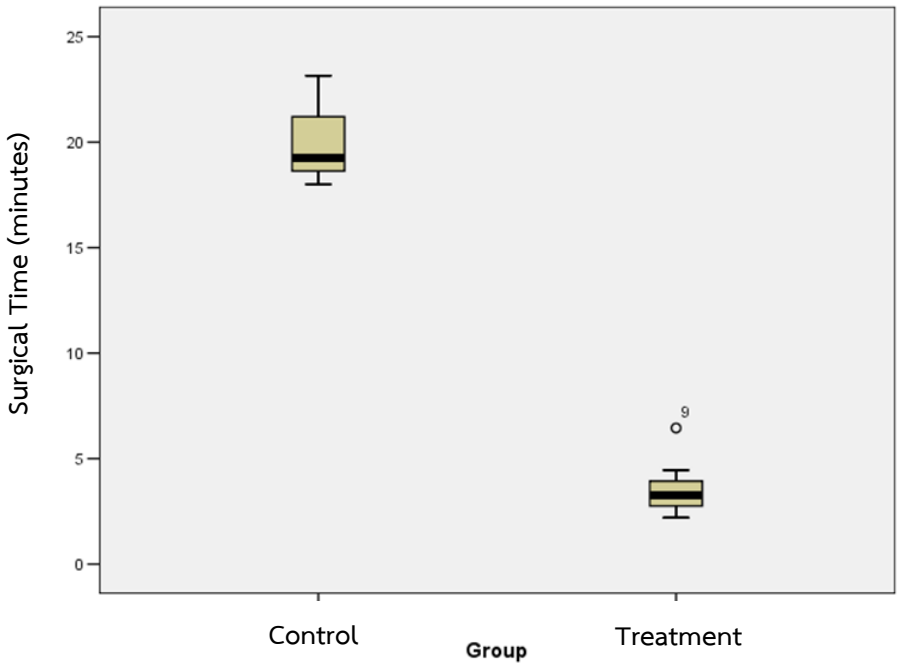

Figure 9 Box plot graph of the surgical time. There was the evidence of statically significant difference between the groups *(p<0.05).*

Clinical outcomes

Table 3 Number of animals in each graft characteristics.

Group of study	Graft loss	Graft fold	Graft shrinkage
Control (n=3)	0 (0%)	3 (100%)	3 (100%)
Treatment (n=7)	1* (14.29%)	1** (14.29%)	0 (0%)

* The incidence was observed on day 3

** The incidence was observed on day 6

Table 4 Number of animals in each ocular complication.

Day of observation	Group of study	Blepharitis	Purulent OD	Epiphora	Hypopyon
D0	Control	0	0	0	0
	Treatment	0	0	0	0
D3	Control	3 (100%)	0	1* (33.33%)	0
	Treatment	0	0	0	0
D6	Control	3 (100%)	0	1* (33.33%)	0
	Treatment	0	0	0	0
D9	Control	3	1* (33.33%)	1* (33.33%)	1* (33.33%)
	Treatment	0	0	0	0
D12	Control	3 (100%)	1* (33.33%)	1* (33.33%)	1* (33.33%)
	Treatment	0	0	0	0

* The complications were observed on the same eye.

In the control group, all membranes were not well attached on the corneal surface throughout the study (Figure 10). They had bulging edges between stitches. The membranes were shrinkage and folded during the follow-up time (14 days). Opacification of the cornea was indicated on the sutured site on day 6 in all animals. The rabbits showed ocular pain and discomfort signs; blepharospasm, photophobia and Epiphora (Figure 11). On day 9, one eye developed hypopyon and severe blepharitis, conjunctivitis and purulent ocular discharge. A peripheral corneal vascularization was observed on day 9 from the limbus in the entire control group (Table 3 and 4).

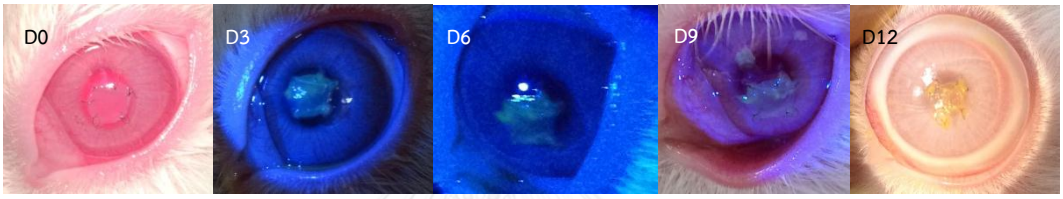

Figure 10 The rabbit cornea in control group on day 0, 3, 6, 9 and 12 post-operatively. Fluorescein staining was positive until day 12 and. Graft shrinkage was first observed on day 3, graft folding and opacification on day 6 and 9 respectively.

Figure 11 Clinical signs in the control group. Purulent ocular discharge and epiphora (A). Severe blepharitis and blepharospasm (B). Hypopyon on day 9 and cornea turned hazed (C).

In the treatment group, 5 by 7 membranes were securely attached and well spread on the corneal surface. One of the membranes sloughed off on day 3 and another one folded on day 6 (Table 3). The grafts showed mild haze and mild shrinkage. The animals showed less ocular pain and discomfort than the control group; mild blepharitis was observed in 3 by 6 rabbits and no peripheral corneal vascularization was observed (Table 4).

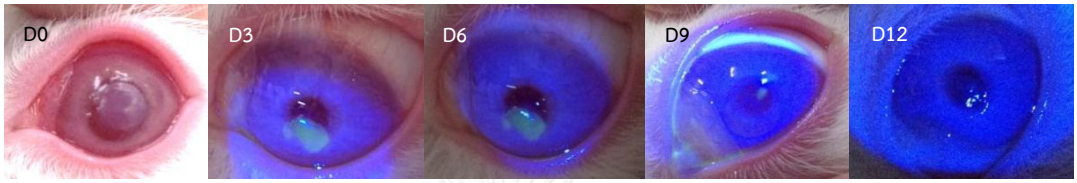

Figure 12 The rabbit cornea in treatment group on day 0, 3, 6, 9 and 12 post-operatively. Fluorescein staining was negative on day 12. The graft well attached and spread on the cornea and the cornea was clear throughout the follow-up time.

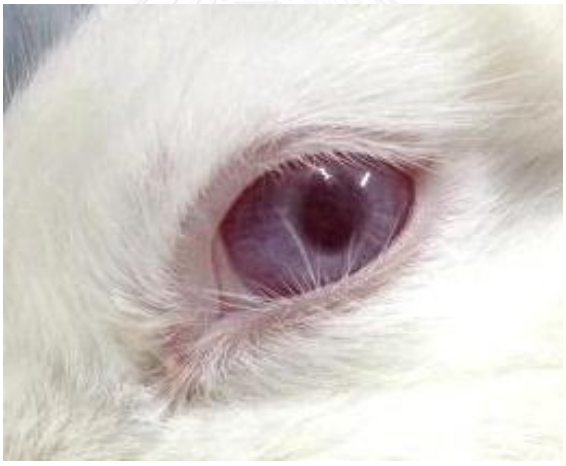

Figure 13 The eye in the treatment group. The cornea was clear and no signs of ocular discomfort and inflammation. The graft could be seen on the corneal surface.

Tear production

Mean tear production measured by Schirmer tear test (STT) in the control group was 10.99±2.03 mm/min and 10.93±1.33 mm/min in the treatment group. Median of the control group was greater than the treatment group; however, there was no a statistically significant difference between the groups (*p>0.05*) (Figure 14).

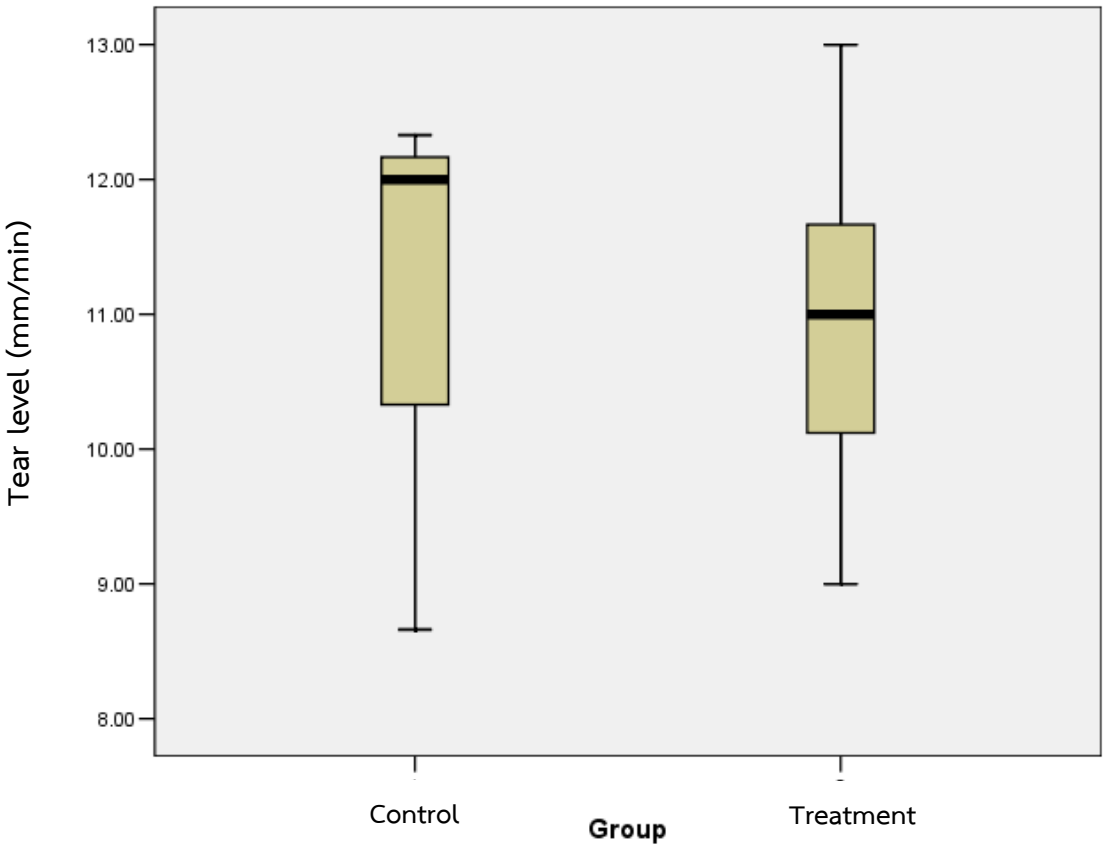

Figure 14 Box plot graph of tear level measured by STT strip. There was no statically significant difference between 2 groups *(p>0.05).*

Duration of Epithelialization

In the treatment group, one eye was excluded because of graft loss. Mean duration of epithelialization of the control group was 11.67±2.52 days and 8.50±2.95 days in the treatment group. Statistically significant difference was not evident between 2 groups *(p>0.05)* (Figure 15).

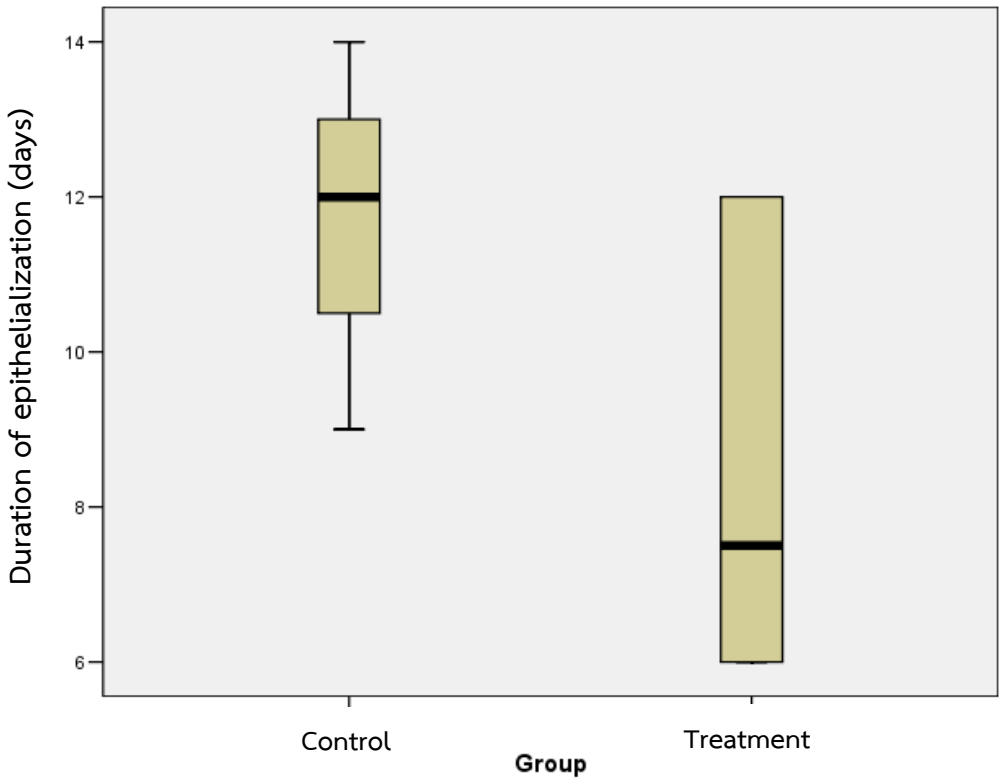

Figure 15 Box plot graph of duration of epithelialization; There was no statically significant difference between the groups *(p>0.05)*.

Ocular pain score

Mean rank of pain score in the control group was 8.33 and 4.29 in the treatment group by Mann-Whitnet U test. There was no statistically significant difference between two groups (*p>0.05*) (Table 5).

Corneal clarity score

Mean rank of corneal clarity score in the control group was 9.00 and in the treatment group was 4.00 by Mann-Whitnet U test. Statistically significant difference was evident in both groups *(p<0.05)* (Table 5).

Table 5 Mean rank of pain score and corneal clarity score.

Group of study	Pain score	Corneal clarity score*
Control (n=3)	8.33	9.00
Treatment (n=7)	4.29	4.00

* Statistically significant difference *(p<0.05)*

Histopathological and Immunohistochemical findings

In the control group, the corneal stromal layers showed the granuloma of the suture material-corneal tissue reaction with infiltration of mononuclear cells and phagocytic cells around the reaction. The grafts were extensively swelled and not securely attached on the corneal wound surface. The epithelial layers were detected by H&E, Masson's trichrome staining and pan-cytokeratin antibody immunostaining. One eye showed the bulging edge resulting in the massively accumulation of the novel epithelium and found some focals of the epithelium beneath the graft. Multilayer of the epithelium covered the membrane unorganized and found thin epithelial layers ran beneath the graft. In one eye of the control group, massively infiltration of neutrophils, macrophages and monocytes was observed in the ciliary body, iridocorneal angle and the anterior chamber with the fibrin precipitation on the endothelium. The corneal-associated lymphoid tissue (CALT) was active in all suture-fixated eyes. The fibroblasts migrated into the membrane and produced the fibrous tissue in all control groups' eyes, some of the keratinocytes started to produce novel layers of collagen fiber. However, the collagenous stromal layers showed the stromal edema and infiltrated with inflammatory cells (Figure 16, 17, 18 and 19).

In the treatment group, the histopathological study showed well adhering of the membranes on the corneal surface in all eyes. Some portion of the grafts was degraded with infiltration of the fibroblasts and keratocytes. A stromal edema and some of the inflammatory cells were found in the corneal stroma fewer than in the control eyes. The persistence of bubaline fibrin glue was detected as pink in the Masson's trichrome staining. The re-epithelialized layers showed 2 to 6 layers of well-organized stratified epithelium covered the graft, that seen in the pan-cytokeratin immunostaining. In 3 by 7 eyes, there was an accumulation of the epithelium before going extended on the surface of the membrane; however, it was less than that in the control group. The fibroblasts were found migrating into the graft and formed the fibrous tissue was similar in the control group (Figure 20, 21 and 22).

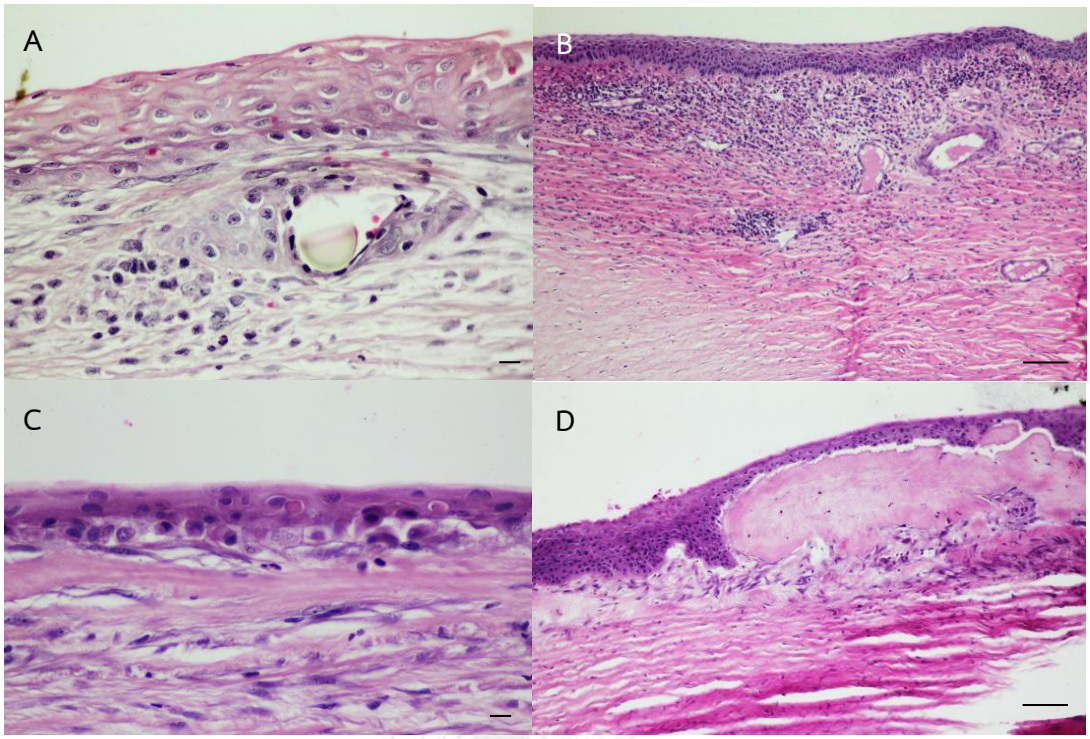

Figure 16 Cornea and limbus in the control group.

A. Suture material surrounded by mononuclear cells.

B. CALT is active, increase number of lymphocytes.

C. Unorganized stratified epithelium on the graft.

D. Graft edema and bulging with epithelial cells accumulation and focal of epithelium found beneath the graft.

H&E stain, A & C; Bar = 10 μm and B & D; Bar = 100 μm.

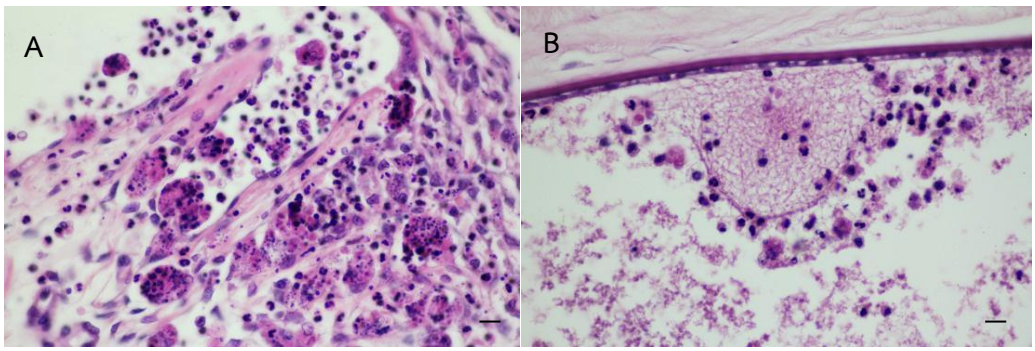

Figure 17 Anterior chamber in the control group**.**

A. Neutrophils, monocytes and macrophages infiltrate the iridocorneal angle.
B. Fibrin precipitation, neutrophils and protein debris present in the anterior chamber.

H&E stain, Bar = 10 μm.

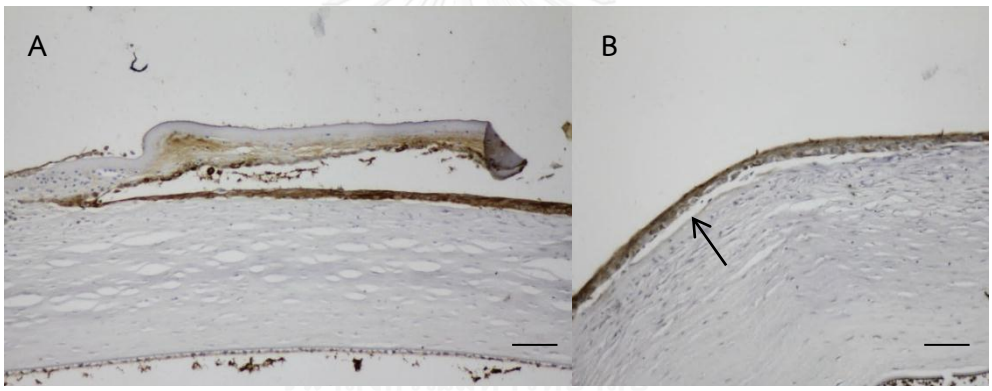

Figure 18 Corneal surface and epithelial sheet in the control group**.**

A. Partial detached graft with the epithelium sheet migrates underneath.
B. The epithelium sheet was slightly separated from the corneal surface (arrow).

Immunostained with pan-cytokeratin antibody, Bar = 100 μm.

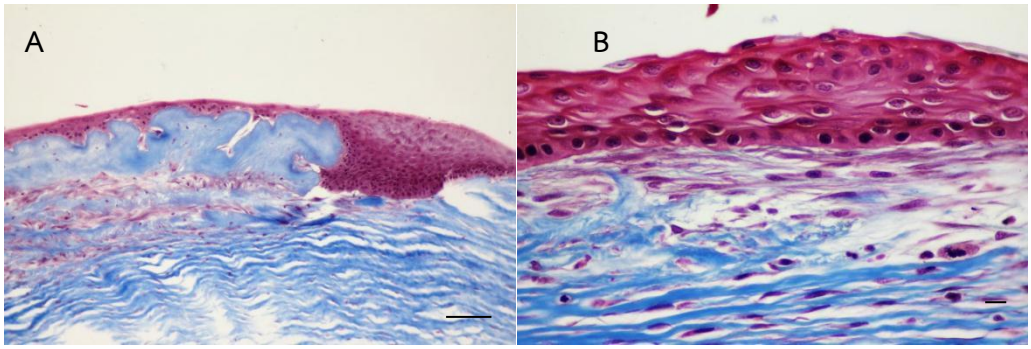

Figure 19 Corneal surface and the graft in the control group**.**

A. Young fibroblasts migrated into the membrane and started to produce fibrous tissue.
B. The epithelial layers form unorganized pattern and there are young fibroblasts underneath.

Masson's Trichrome stain, A; Bar = 100 μm and B; Bar = 10 μm.

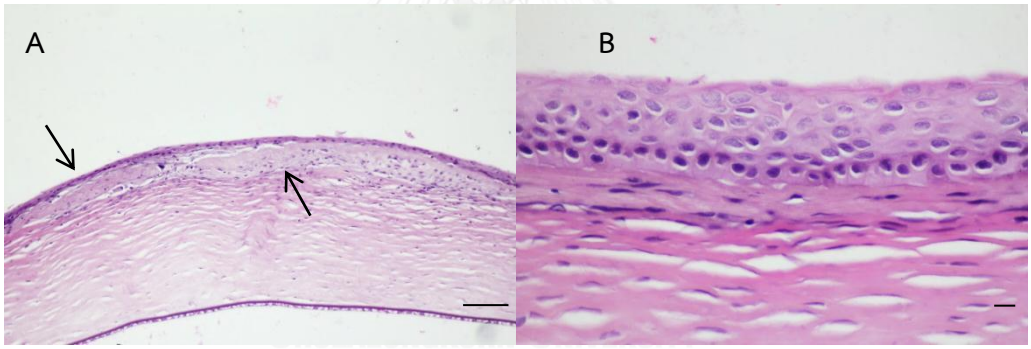

Figure 20 Cornea in the treatment group**.**

A. The margin of the graft is firmly attached on the surface (right arrow) with thin layers of the epithelium move on the graft (left arrow).
B. Layers of the epithelium run smoothly over the graft and well organized. There are some fibroblasts in the membrane.

H&E stain, A; Bar = 100 μm and B; Bar = 10 μm.

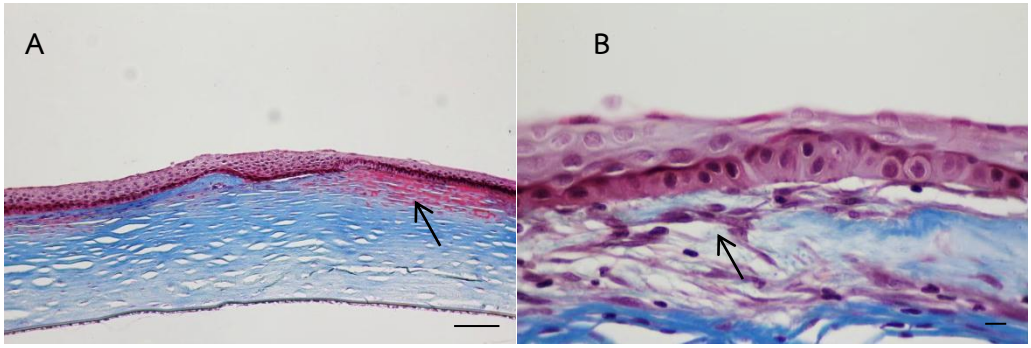

Figure 21 Cornea in treatment group**.**

A. The graft is firmly attached on the corneal surface with epithelial layers are well covered on the graft. Young fibroblasts started to migrate to the wounded site (arrow).
B. Young fibroblasts (arrow) migrate into the membrane and started to produce a novel fibrous tissue.

Masson's Trichrome stain, A; Bar = 100 μm and B; Bar = 10 μm.

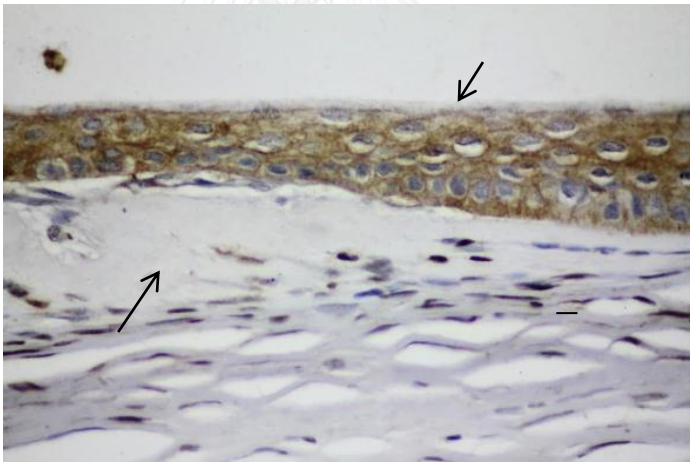

Figure 22 Epithelial layers on the corneal surface in the treatment group**.** The epithelium (right arrow) well covers on the graft (left arrow). Immunostained with pan-cytokeratin antibody; Bar = 10 μm.

CHAPTER V
DISCUSSION

Corneal ulcer is one of the most common defects of the eye. It is multifactorial diseases; intrinsic and extrinsic factors. Some of them are extensively large and complicated to manage with only medications; therefore, surgical correction has to be performed. Autograft such as conjunctival flap is one of the most popular methods to correct the corneal ulceration; however, xenograft, HAMT, provides desirable outcomes (Dua et al., 2004; Gomes et al., 2005; Baradaran-Rafii et al., 2007).

Commercial bovine-blood fibrin glue has been reported by many studies that could reduce surgical time, limitations and complications from using suture materials on human amniotic membrane fixation (Szurman et al., 2006; Vichare et al., 2013). However, buffalo blood provides higher level of fibrinogen the other animals; it would offer better efficacies than that from bovine blood.

The whole blood was sent for blood culture which bacterial growth was not found. It can be assumed that collecting technique was successfully aseptic. All of the materials and equipment used in the process of glue producing were sterilized and the extract; bubaline fibrin glue, was sent for bacterial culture which result was no bacterial growth. This bubaline fibrin glue might safe enough to be used in this study.

Moreover, Banyatworakul et al. (2016) reported that bubaline fibrin glue had antimicrobial activity against 6 strains of bacteria from both Gram's positive and Gram's negative groups by showing clear zone in disc diffusion method.

The concentration of fibrinogen extract was 700 times higher than that in whole blood. This method; cryoprecipitation which modified from Thorn et al. (2004), was a satisfactory homemade glue producing process and might be applied widely. However, the glue could last long only one month; therefore, the glue should be used within a month before the occurrence of fibrinogen concentration reduction.

By this study, homemade bubaline fibrin glue might hold HAM better than the suture materials. Percentage of graft folding and shrinkage in the control group were higher than the treatment group. Although there was a graft slough in the treatment group, another was still well spread and less evidence of graft folding and shrinkage than the control group. Szurman et al. (2006) described that fibrin cross-linked took the first role on fixing the membrane, then covering epithelial sheet on the membrane came to take part.

Suture-fixated HAMT has been reported that it was a time-consuming procedure (Panda et al., 2009); this study would showed that bubaline fibrin glue can eliminate this disadvantage. The surgical time was significantly different ($p<0.05$) between the control and treatment groups. The suture-fixated group took more time than the glue-fixated group because the fibrin glue can be formed and ready to fix the membrane in place within 3.16±1.46 minutes. Comparable to the study of Szurman et al. (2006) using the commercial glue set within 3 minutes. Uy et al. (2005) reported that fibrin glue help shorten the average surgical time in pterygium excision in human ($p<0.001$). As well as Mahdy and Wagieh (2011) reported that fibrin glue could reduce the surgical time significantly. Therefore, it would be stated that use of fibrin glue could reduce the surgical time of HAMT.

One animal in the treatment group had surgical time of 6.45 minutes. It might be because of glue reduce the fixation property due to the animal was performed surgery as the last; therefore the glue was thawed more than 3 hours before used. Most commercial products of fibrin sealant are recommended to be used within 2 hours after thawing in order to keep the best qualities of the glue. Therefore, it could be assumed that the bubaline fibrin glue should be used within 2 hours after being thawed to control the fixation property. This occurred with the rabbit number T6 in the treatment group which the graft could not be securely attached and sloughed off on day 3. It might be because the glue was thawed for a longer time than it should be.

However, Fang and Baratz (2004) used the commercial fibrin sealant; Tisseel®, to attach preserved HAM on the corneal surface and reported that 100% of the membranes sloughed off from the corneal surface (4/4 in the first phase and 2/2 in the second phase). Thus, it might be assumed that the homemade bubaline fibrin glue appears superior to the commercial bovine fibrin glue in term of fixation efficacy.

Complications of using the suture materials were observed in the control group much more than the treatment. Accordingly, the bubaline fibrin may help reduce the complications and provide the better outcomes than suture materials.

In the control group; blepharitis, blepharospasm, purulent ocular discharge and anterior uveitis were noted which was consistent with many studies stated that suture materials could be the cause because of suture-tissue reaction. (Szurman et al., 2006; Panda et al., 2009; Vichare et al., 2013). By histopathological study, the microphotographs were consistent with the clinical signs. One eye of the control group developed sterile hypopyon which was indicated by non-toxic neutrophils. It

could be because of the suture material inducing inflammation reaction and activated CALT. The pro-inflammatory cytokines were released and attracted inflammatory cells to the site of inflammation and resulting in anterior uveitis eventually. There were better clinical signs in the treatment group. Mild Blepharitis and blepharospasm were observed only a first few6 days post-operatively.

Duration of fluorescein staining was used for measuring the re-epithelialization time. Although there was not significantly different between the 2 groups, raw data showed the trend that the treatment group has a novel epithelialization faster than in the control group. Because of the glue, the membrane can closely attach on the corneal surface than the suture-fixated group and novel epithelial layers can easily migrate onto the membrane. This result is consistent with the study of Szurman et al. (2006). Having epithelium underneath the graft in the control group, it could speed up graft dissolving due to collagenases present on the ocular surface, while the fibrin glue covered over the graft forms a thin, porous layer allowing nutrients and beneficial molecules transport through it to enrich the graft-host interface.

Tear production based on STT measurement was not significantly different between the control and treatment groups. Although, mean of tear level of both group was not significantly different, the trend of tear production in the control group appeared greater than the treatment group. It might be because of suture-inducing irritation. However, from the study of Whittaker and Williams (2015) which had studied the lacrimation characteristics of normal New Zealand White rabbits' eye reported the normal tear production was 7.58 ± 2.3 mm/minute which was less than both groups from this study. When the corneal surface is breakdown, tearing reflex will be upregulated in the early neurological inflammatory phase and tear plays the major role in ocular surface healing which it is enrich of the beneficial proteins; such as Defensin (Zhou et al., 2007). Because of the need of tear for corneal healing, both groups have higher mean of tear production than the normal eyes.

Tear can disrupt fibrin crosslink because of the proteolytic enzymes which significantly increased in damaged cornea (Ollivier et al., 2007). Tranexamic acid added into this bubaline fibrin glue can protect the fibrin crosslink. It has an antifibrinolytic effect through the reversible blockade of lysine binding sites on plasminogen molecules (Dunn and Goa, 1999). Moreover, amniotic membrane can inhibit protease activity (Szurman et al., 2006). In fact, epsilon-aminocaproic acid also has the effect, but tranexamic acid provides 6 to 10 times more potent than the epsilon-aminocaproic acid in terms of binding to the plasminogen and plasmin (Dunn and Goa, 1999).

Resulting from the study, the use of the bubaline fibrin glue to fix the membrane on the wounded corneal surface is satisfactory in both terms of attachment property and reduction of disadvantages from using suture materials.

In conclusion, this study demonstrated that the fibrin glue derived from buffalo blood could help holding the HAM lying well on the corneal than the suture fixation, moreover the novel method provide better outcomes; clarity of the cornea, surgical time, duration of re-epithelialization, and less complications; Blepharitis, conjunctivitis, ocular discharge and anterior uveitis, than the conservative method.

However, the study of using the bubaline fibrin glue should be investigated further; increase sample size, expand the follow up time, and develop the effectiveness and efficacy of bubaline fibrin glue. Moreover, the glue should be deeply examined the quantities and qualities of the fibrinogen protein, thrombin protein and the growth factors.

Bubaline fibrin glue would be the choice of surgical correction in Veterinary Ophthalmology due to the less surgical time, desirable outcomes and fewer complications.

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APPENDIX

Appendix 1 Table of the raw data of the surgical time (minutes)

Group	Number of animals and Sex*	Surgical time (min)
Control	C1 (F)	18:00
	C2 (M)	23:15
	C3 (M)	19:26
Treatment	T1 (F)	03:22
	T2 (M)	03:27
	T3 (F)	02:20
	T4 (M)	02:29
	T5 (M)	04:45
	T6 (F)	06:45
	T7 (M)	03:42

* F = Female, M = Male

Appendix 2 Table of the raw data of graft characteristics

Number	Graft edema[3]	Graft shrinkage[33]
C1	1	1
C2	1	1
C3	1	1
T1	0	0
T2	0	1
T3	0	0
T4	0	0
T5	1	1
T6	/	/
T7	1	1

[3] 0 = Graft was not edema
1 = Graft was edema
/ = Missing data

[33] 0 = Graft was not shrinking
1 = Graft was shrinking
/ = Missing data

Appendix 3 Table of the raw data of the clinical outcomes

Number	Corneal clarity score[†]	Ocular pain score[††]	Epithelialization (days)	Tear level (mm/min)
C1	3	1	14	8.6
C2	3	2	9	12.3
C3	2	1	12	12.0
T1	1	0	6	11.3
T2	1	1	6	9.0
T3	1	0	12	12.0
T4	1	0	9	11.0
T5	1	1	12	10.2
T6	1	0	1	10.0
T7	1	0	1	13.0

[†] **1** = Clear cornea

2 = Mild haze cornea, all of underlying structures are easily seen

3 = Moderate haze cornea, some of underlying structures are seen

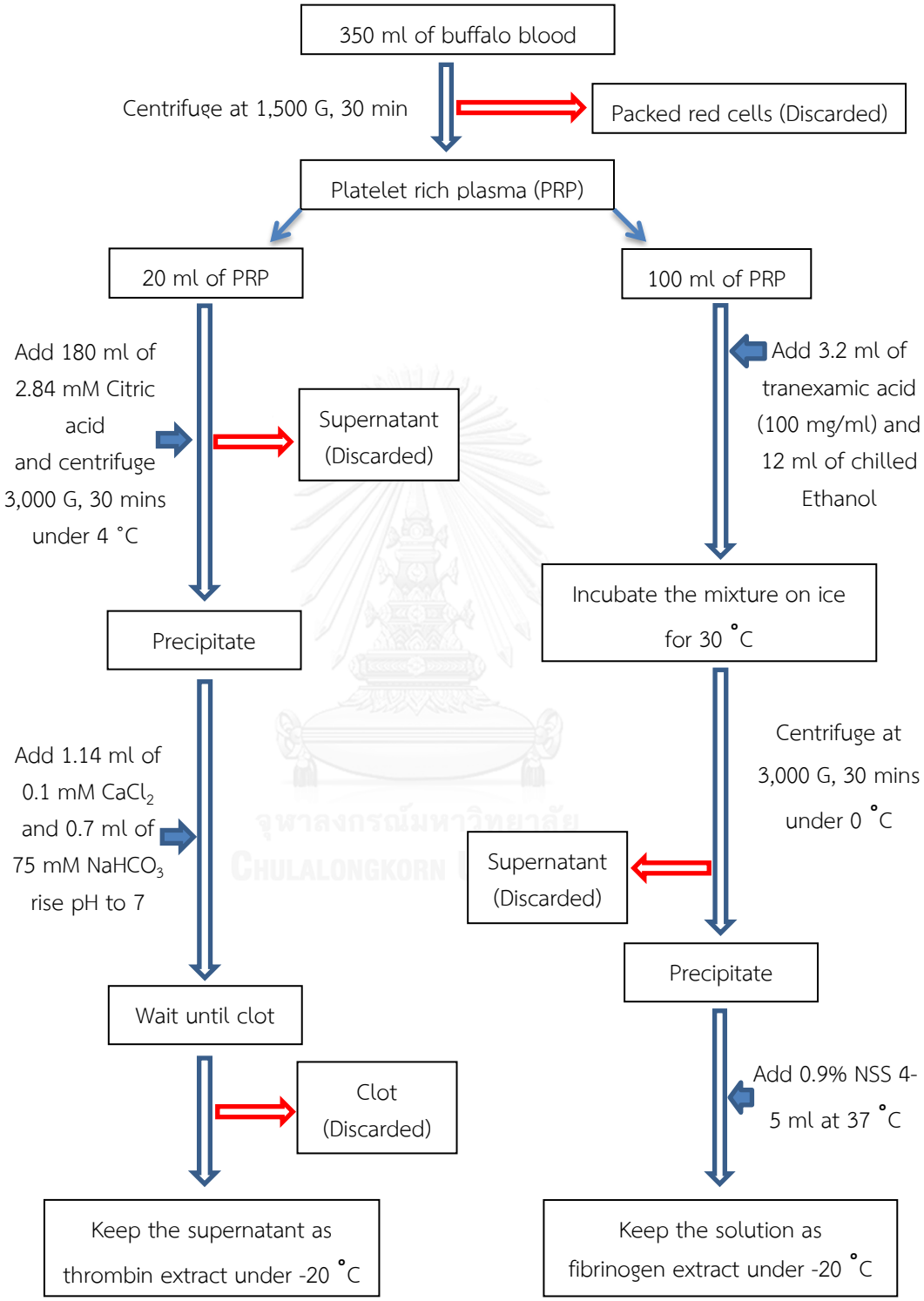
4 = Severe haze cornea, none of underlying structures are seen

[††] **0** = Normal present

1 = Narrowing of palpebral fissure

2 = Tightly close of the eyelid and wrinkle may be visible

Appendix 4 Flow chart of preparation of bubaline fibrin glue



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